Exhibit 5

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Expression of the α(1,3)Fucosyltransferase Fuc-TVII in Lymphoid Aggregate High Endothelial Venules Correlates with Expression of L-Selectin Ligands*

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Lymphocyte homing to lymph nodes and Peyer's patches is mediated, in part, by adhesive interactions between L-selectin expressed by lymphocytes and L-selectin ligands displayed at the surface of the cuboidal endothelial cells lining the post-capillary venules within lymphoid aggregates. Candidate terminal oligosaccharide structures thought to be essential for effective L-selectin ligand activity include a sulfated derivative of the sialyl Lewis x tetrasaccharide. Cell type-specific synthesis of this oligosaccharide is presumed to require one or more α(1,3)fucosyltransferases, operating upon common 3′-sialylated and/or sulfated *N*-acetyllactosamine-type precursors. The identity of the α(1,3)fucosyltransferase(s) expressed in cells that bear L-selectin ligands has not been defined. We report here the molecular cloning and characterization of a murine α(1,3)fucosyltransferase locus whose expression pattern correlates with expression of high affinity ligands for L-selectin. *In situ* hybridization and immunohistochemical analyses demonstrate that this cDNA and its cognate α(1,3)fucosyltransferase are expressed in endothelial cells lining the high endothelial venules of peripheral lymph nodes, mesenteric lymph nodes, and Peyer's patches. These expression patterns correlate precisely with the expression pattern of L-selectin ligands identified with a chimeric L-selectin/IgM immunohistochemical probe and by the high endothelial venule-reactive monoclonal antibody MECA-79. Transcripts corresponding to this cDNA are also detected in isolated bone marrow cells, a source rich in the surface-localized ligands for E- and P-selectins. Sequence and functional analyses indicate that this murine enzyme corresponds to the human Fuc-TVII locus. These observations suggest that Fuc-TVII participates in the generation of α(1,3)fucosylated ligands for L-selectin and provide further evidence for a role for this enzyme in E- and P-selectin ligand expression in leukocytes.

Cell adhesion events between leukocytes and endothelial cells operate to facilitate the exit of blood leukocytes from the vascular tree. The selectin family of cell adhesion molecules and their counter-receptors function early in this process, mediating transient adhesive contacts between leukocytes and the endothelial cell monolayer. These selectin-dependent adhesive contacts, together with shear forces impinging upon the leukocyte, cause the leukocyte to "roll" along the endothelial monolayer. Leukocyte rolling, in turn, facilitates subsequent events that include leukocyte activation, firm leukocyte-endothelial cell attachment, and transendothelial migration (1, 2).

E- and P-selectins, expressed by activated vascular endothelial cells, recognize glycoprotein counter-receptors displayed by leukocytes. Each of these selectins can operate to mediate leukocyte rolling in the context of inflammation. L-selectin has also been implicated in mediating leukocyte adhesion to activated vascular endothelium through interactions with an as yet poorly understood endothelial cell ligand (3, 4). By contrast, lymphocyte L-selectin recognizes glycoprotein counter-receptors displayed by specialized cuboidal endothelial cells that line high endothelial venules (HEV)[1] within lymph nodes and Peyer's patches. L-selectin-dependent adhesive interactions in this context operate to facilitate trafficking of lymphocytes (lymphocyte "homing") to such lymphoid aggregates.

The NH_2-terminal C-type mammalian lectin domain common to each of the three selectin family members mediates cell adhesion through calcium-dependent interactions with specific oligosaccharide ligands, displayed by leukocytes (E- and P-selectin ligands) (2, 5) or by HEV (L-selectin) (6). Physiological ligand activity for E- and P-selectins is critically dependent on the expression of a nonreducing terminal tetrasaccharide termed sialyl Lewis x (sLe^x) [NeuNAcα2,3Galβ1,4 (Fucα1,3)-GlcNAc-R] (5) and/or its difucosylated variant (7). However, E- and P-selectins recognize this oligosaccharide in different contexts. P-selectin-dependent cell adhesion is optimal when sLe^x is displayed by serine and threonine-linked oligosaccharides residing on a specific protein termed P-selectin glycoprotein ligand 1 (PSGL-1) (8, 9). sLe^x-modified P-selectin glycoprotein ligand 1 also appears to represent a high affinity counter-receptor for E-selectin (10, 11). A distinct leukocyte glycoprotein termed E-selectin ligand 1 (ESL-1) (12) and its α(1,3)fucosylated, asparagine-linked oligosaccharides may also function as an E-selectin counter-receptor.

Physiological L-selectin counter-receptors on HEV are rep-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U45980.

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[1] The abbreviations used are: HEV, high endothelial venule(s); sLe^x, sialyl Lewis x tetrasaccharide; CHO, Chinese hamster ovary; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate.

resented by the glycoproteins GlyCAM-1 (13), CD34 (14), and MAdCAM-1 (15). Biochemical studies indicate that L-selectin ligand activity of these molecules is also critically dependent upon post-translational modification by glycosylation. Early studies documented a requirement for sialylation and sulfation (16), implied a requirement for α(1,3)fucosylation, and indicated that these modifications are components of serine- and/or threonine-linked glycans. More recent oligosaccharide structural analyses extend this work and imply that high affinity L-selectin ligand activity may depend upon a sulfated variant of the sLe^x determinant, NeuNAcα2,3($SO_4$6)Galβ1,4(Fucα1,3)GlcNAc-R (17–19).

Expression of sLe^x is determined by cell lineage-specific expression of one or more α(1,3)fucosyltransferases (20). These enzymes utilize the donor substrate GDP-fucose and catalyze a transglycosylation reaction involving the addition of α1,3-linked fucose to a common 3′-sialyl *N*-acetyl-lactosamine precursor. It can be presumed that expression of the sulfated variant of sLe^x also depends upon lineage-specific expression of α(1,3)fucosyltransferase activities operating on sulfate-modified 3′-sialyl *N*-acetyl-lactosamine precursors or that create sLe^x moieties modified subsequently by sulfation.

The identity of the α(1,3)fucosyltransferase(s) responsible for selectin ligand expression in leukocytes is not well defined, and HEV-specific α(1,3)fucosyltransferases have not been described. To date, five different human α(1,3)fucosyltransferases have been cloned (21–28). Northern blot and molecular cloning analyses imply that two of these, termed Fuc-TIV (24–26) and Fuc-TVII (27, 28), are expressed in leukocytic cells and represent candidates for critical participation in selectin ligand expression. The role of Fuc-TIV (also known as ELAM-1 ligand fucosyl transferase) in this process is not clear, however. Although Fuc-TIV/ELAM-1 ligand fucosyl transferase is able to efficiently utilize nonsialylated *N*-acetyl-lactosamine precursors to direct expression of the Le^x moiety (24, 26), this enzyme cannot determine sLe^x expression in all cellular contexts (29), and its ability to do so in leukocytes or in leukocyte progenitors has not been demonstrated. By contrast, Fuc-TVII is apparently able to determine sLe^x expression in all mammalian cellular contexts examined, where sLe^x synthesis is biochemically possible (27, 28). Neither enzyme has been tested for its ability to participate in the synthesis of L-selectin ligands represented by sulfated sLe^x determinants.

We report here the isolation and characterization of murine cDNAs and genomic sequences encoding an α(1,3)fucosyltransferase with primary sequence similarity and catalytic properties analogous to those assigned to the human Fuc-TVII α(1,3)fucosyltransferase (27, 28). This murine locus generates alternatively spliced transcripts that differ in their respective abilities to encode α(1,3)fucosyltransferase activity. Expression of this locus is restricted largely to E- and P-selectin ligand-rich bone marrow cells (30), where it may participate in the synthesis of these ligands. Transcripts derived from the Fuc-TVII locus and the corresponding α(1,3)fucosyltransferase accumulate to substantial levels in the endothelial cells lining the HEV of peripheral and mesenteric lymph nodes and of Peyer's patches. The localized and abundant expression of this α(1,3)fucosyltransferase in HEV, when considered together with the α(1,3)fucosylated oligosaccharides proposed as HEV ligands for L-selectin, imply a key role for this enzyme in the biosynthesis of L-selectin ligands.

EXPERIMENTAL PROCEDURES

Cell Culture—The sources and growth conditions for COS-7 cells (31), CHO-Tag cells (32), and cultured murine blood cell lines (B cell line S107 (33)); T cell line EL4 (34); B cell hybridoma line TH2.54.63 (35); B cell hybridoma line 180.1 (36); Friend murine erythroleukemia cell line MEL (37, 38); macrophage cell line RAW264.7 (39, 40); macrophage cell line P388D_1 (41); and the cytotoxic T-cell line 14-7fd (42, 43).

Antibodies—The sources of the monoclonal antibodies used here have been described previously (anti-Lewis x/anti-SSEA-1 (44); anti-H and anti-Lewis a (31); anti-sialyl Lewis x/CSLEX (45); anti-sialyl Lewis a (46); anti-VIM-2 antibody (47); and fluorescein-conjugated goat anti-mouse IgM and IgG antibodies; Sigma). MECA-79 (48) was the generous gift of Drs. Louis Picker and Eugene Butcher (Stanford University).

cDNA Cloning—Mouse Fuc-TVII cDNAs were isolated from a cDNA library constructed from the mouse cytotoxic T cell line 14-7fd (32), using colony hybridization procedures (49) and a segment of the mouse Fuc-TVII gene (see Fig. 2) corresponding to nucleotides 2053–2285.

Murine Genomic Library Screening—Approximately 1.0 × 10^6 recombinant lambda phage from a genomic library prepared from mouse 3T3 cell DNA (Stratagene) were screened by plaque hybridization using a 324-base pair segment of the human Fuc-TIII gene (nucleotides 571–894) and low stringency hybridization procedures described previously (31, 50). DNA from a phage with a unique restriction pattern was digested with *Sac*I, and a 2.6-kilobase pair fragment that cross-hybridized with the human Fuc-TIII probe was gel purified and cloned into the *Sac*I site of pTZ19R (Pharmacia Biotech Inc.). A representative subclone containing a single insert was designated pMFuc-TVII. The DNA sequence of the 2.6-kilobase pair insert was determined by the dideoxy chain termination method (51) using T7 DNA polymerase (Sequenase, U. S. Biochemical Corp.), and oligonucleotide primers were synthesized according to flanking plasmid sequences. Sequence data were used to design additional synthetic primers, which were then utilized to sequence the remaining portion of the *Sac*I insert in pMFuc-TVII. Sequence analysis was performed using the sequence analysis software package of the University of Wisconsin Genetics Computer Group (52) and the MacVector version of the IBI Pustell Sequence Analysis Software package (IBI). Sequence alignments were assembled with the Gap function of the Genetics Computer Group package.

Transfection and Analysis of COS-7 Cells and CHO-Tag Cells—COS-7 cells were transfected with various Fuc-TVII expression vectors using a DEAE-dextran transfection procedure previously described (21, 49). CHO-Tag cells were transfected with plasmid DNAs using a liposome-based reagent (*N*-[2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate, Boehringer Mannheim) as modified previously (32).

Transiently transfected cells were harvested 72 h after transfection and were stained with monoclonal antibodies diluted in staining medium as described previously (22). Anti-Lewis a, anti-H, and anti-sialyl Lewis x antibodies were used at 10 μg/ml. Anti-Lewis x was used at a dilution of 1:1000. Anti-sialyl Lewis a was used at a dilution of 1:500. Anti-VIM-2 antibody was used at a dilution of 1:200. Cells were then stained with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM or anti-mouse IgG and subjected to analysis on a FACScan (Becton-Dickinson) as described previously (22). Cells were also co-transfected with the plasmid pCDM8-CAT (32), and extracts prepared from these cells were subjected to chloramphenicol acetyltransferase activity assays (32) to allow for normalization of flow cytometry and Western blot data to transfection efficiency.

Fucosyltransferase Assays—COS-7 cells transiently transfected with Fuc-TVII expression vectors were harvested 72 h after transfection, and extracts were prepared from these cells exactly as described previously (21, 23). These extracts were subjected to α(1,3)fucosyltransferase assays (22) assembled in a total volume of 20 μl. Reaction mixtures contained 3 μM GDP-[^{14}C]fucose, 20 mM acceptor (*N*-acetyllactosamine, lactose, lacto-*N*-biose I, 2′-fucosyllactose (Sigma), or 3′ sialyl *N*-acetyllactosamine (Oxford Glycosystems)), 50 mM cacodylate buffer, pH 6.2, 5 mM ATP, 10 mM L-fucose, 15 mM $MnCl_2$, and a quantity of cell extract protein sufficient to yield approximately linear reaction conditions (consumption of less than 15% of the GDP-fucose substrate) during the course of the reaction (1 h). Control reactions were prepared by omitting the acceptor in the reaction mixture, and values obtained with these reactions were subtracted from the corresponding acceptor-replete reaction. This background radioactivity reproducibly represented less than 1% of the total radioactivity in the assays and corresponds to free [^{14}C]fucose present in the GDP-[^{14}C]fucose as obtained from the manufacturer. Identical enzyme preparations were used in assays for the determination of enzyme activity with different acceptor substrates.

Reactions containing neutral acceptors (*N*-acetyllactosamine, lactose, lacto-*N*-biose I, 2′-fucosyllactose, all from Sigma) were terminated by the addition of 20 μl of ethanol and 560 μl of water. Samples were centrifuged at 15,000 × *g* for 5 min and a 50-μl aliquot was subjected to scintillation counting to determine the total amount of radioactivity in the reaction. An aliquot of 200 μl was applied to a column containing 400 μl of Dowex 1 × 2–400, formate form (21, 23). The column was

washed with 2 ml of water, and the radioactive reaction product not retained by the column was quantitated by scintillation counting. Reactions with the acceptor Neu*NA*cα2→3Galβ1→4GlcNAc (Oxford Glycosystems, Inc.) were terminated by adding 980 μl of 5.0 mM sodium phosphate buffer, pH 6.8. Samples were then centrifuged at 15,000 × *g* for 5 min, and a 500-μl aliquot was applied onto a Dowex 1 × 8–200 column (1 ml) prepared in the phosphate form. The reaction product was collected and quantitated as described previously (22).

Generation of Rabbit Anti-Fuc-TVII Antibody—PCR was used to amplify a segment of the murine Fuc-TVII gene corresponding to the enzyme's "stem" and catalytic domains (53), using PCR primers corresponding to base pairs 2194–2224 and 3053–3085 (Fig. 2, 5′ primer, gcgcggatccCACCATCCTTATCTGGCACTGGCCTTTCACC, and 3′ primer, gcgcggatccAGTTCAAGCCTGGAACCAGCTTTCAAGGTCTTC; *Bam*HI sites are *underlined*). PCR was completed using 20 rounds of amplification consisting of a 1.5-min 94 °C denaturation step and a 2.0-min 72 °C annealing/extension step. The PCR product was subsequently cloned into the *Bam*HI site of the T7 *Escherichia coli* expression vector pET-3b (54). The insert in one clone (termed pET-3b-Fuc-TVIIstem/cat) containing a single insert in the correct orientation was sequenced to confirm that no errors were introduced during DNA amplification. The recombinant Fuc-TVII fusion protein was produced by inducing mid-log phase *E. coli* (BL21 Lys S) carrying pET-3b-Fuc-TVIIstem/cat with 0.4 mM IPTG for 3 h (50, 54). The bacteria were subsequently harvested and lysed by freezing and then thawing the bacterial suspension. Bacterial genomic DNA was sheared by sonication, followed by separation of soluble and insoluble material by centrifugation. The Fuc-TVII protein was found in the insoluble fraction, as determined by SDS-polyacrylamide electrophoresis (55).

Recombinant *E. coli*-derived Fuc-TVII was fractionated by SDS-polyacrylamide gel electrophoresis, and segments of the gel containing Fuc-TVII were excised and used subsequently as antigen for rabbit immunizations. Rabbit immunization services were purchased (Pel-Freeze Biologicals, Rogers, AR). Each of three rabbits were initially immunized subcutaneously with a total of approximately 200 μg of Fuc-TVII in pulverized polyacrylamide gel slices mixed with complete Freund's adjuvant. Subsequent immunizations were completed in an essentially identical manner at 14-day intervals, except that antigen was administered in incomplete Freund's adjuvant. Antisera were harvested 10 days following the last of a total of approximately six secondary immunizations.

Antigen Affinity Purification of Anti-Fuc-TVII Antibody—The insert in pET-3b-Fuc-TVIIstem/cat was released by digestion with *Bam*HI and was cloned between the *Bam*HI sites in the *E. coli* expression vector pATH10 (56) to yield a fusion protein derived from the *E. coli* anthranilate synthase sequence, fused in frame to Fuc-TVII sequence. This recombinant fusion protein was expressed in *E. coli* strain DH5α (induction for 6 h with 0.0125% indoleacrylic acid in M9 medium). The bacteria were harvested, washed, and disrupted by treating with lysozyme (3 mg/ml) in 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, with 0.65% Nonidet P-40, and 0.38 M NaCl followed by sonication for 20 s at the maximal microtip setting (Vibracell, Sonics and Materials, Inc., Danbury, CT) (56). Inclusion bodies were washed twice with 50 mM Tris-HCl, pH 7.5, and 5 mM EDTA, were solubilized by heating to 100 °C in 1% SDS, 12 mM Tris-HCl, 5% glycerol, and 1% 2-mercaptoethanol, and were subjected to SDS-polyacrylamide gel electrophoresis. The fractionated proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad) by electroblotting (1 mA/cm^2). The membrane was then blocked for 4–6 h at 4 °C with PBS containing 10% bovine serum albumin and 0.2% Tween-20. A strip of membrane containing the recombinant Fuc-TVII fusion protein was incubated overnight at 4 °C with 0.5 ml of rabbit anti-mouse Fuc-TVII antiserum diluted with 2.5 ml of PBS containing 3% bovine serum albumin and 0.2% Tween-20. The membrane was then washed at room temperature in PBS with 0.05% Tween-20 and sliced into small pieces, and the bound antibody was eluted by incubating the membrane fragments on ice for 10 min in 450 μl of Tris glycine, pH 2.5. The supernatant was collected and immediately neutralized with 100 μl of 1 M Tris-HCl, pH 8.0. The elution procedure was completed a second time, and the two eluates were pooled and used subsequently for immunohistochemical procedures.

Western Blot Analysis—Cell extracts were prepared from transfected COS-7 cells 72 h after transfection. Extracts contained 50 mM Tris-HCl, pH 6.8, 1% SDS, and 10% glycerol. Extracts were boiled for 3 min immediately after preparation and were stored frozen until use. Protein content was determined using the BCA reagent procedure. Extracts were prepared for SDS-polyacrylamide gel electrophoresis by adding dithiothreitol to a final concentration of 0.1 M and bromphenol blue to a final concentration of 0.05%. Samples were then boiled and fractionated by electrophoresis through a 10% SDS-polyacrylamide gel. After electrophoresis, the proteins were electrotransferred to a polyvinylidene difluoride membrane (Bio-Rad). The membrane was rinsed and then blocked for 12–14 h at 4 °C in phosphate-buffered saline, pH 7.4, containing 10% bovine serum albumin and 0.2% Tween 20. The blot was washed at room temperature in phosphate-buffered saline, pH 7.4, with 0.2% Tween 20 and was probed with a 1:200 dilution of antigen affinity purified rabbit anti-Fuc-TVII antibody. The blot was then washed and probed with a 1:2500 dilution of a horseradish peroxidase-conjugated anti-rabbit immunglobulin (Sigma). The blot was then rinsed, exposed to ECL reagent (Amersham Corp.), and subjected to autoradiography.

Northern Blot Analysis—Total RNA was prepared from mouse (FVB/N) tissues and cultured cell lines using published procedures (49). Oligo(dT)-purified poly(A)$^+$ RNA samples were electrophoresed through 1.0% agarose gels containing formaldehyde and were transferred to a nylon membrane (Hybond-N, Amersham Corp.). Northern blots were prehybridized for 2 h at 42 °C in 1 × PE, 5 × SSC, 0.5% sodium dodecyl sulfate, and 150 μg/ml sheared salmon sperm DNA. Blots were hybridized for 18 h at 42 °C in prehybridization solution containing α-^{32}P-labeled 974-base pair *Eag*I–*Eco*RI fragment isolated from the insert in pMFuc-TVII. The *Eag*I site is located at nucleotides 2228–2233, whereas the *Eco*RI site spans base pairs 3202–3207. Blots were stripped in boiling 0.1% SDS and rehybridized with a chicken glyceraldehyde 3-phosphate dehydrogenase probe (57) to confirm that RNA samples were intact and loaded in equivalent amounts (data not shown).

Construction of a Mouse L-selectin/IgM Chimera Histochemical Probe—A mouse L-selectin cDNA (58) was kindly provided by Dr. Mark Siegelman at the University of Texas Southwestern Medical Center (Dallas, TX). The extracellular domain was truncated at the junction of its transmembrane domain with an *Hph*I digest followed by the ligation of an adaptor. A human IgM cDNA containing the CH2, CH3, and CH4 domains (kindly provided by Dr. Ernie Kawasaki, Procept Inc.) was ligated to the adaptor modified end of the L-selectin sequence in a manner that fuses the open reading frame encoding L-selectin to the open reading frame encoding the CH2, CH3, and CH4 domains of human IgM. This fragment was inserted into the vector SRα-PCDM8 immediately downstream of the SRα promoter in the sense orientation with respect to the SRα promoter. This vector was introduced into COS-7 cells using the DEAE-dextran transfection method (21, 49). Medium was harvested from the transfected cells 3 days after the transfection and was replaced with fresh medium (Dulbecco's modified Eagle's medium, 10% fetal calf serum, P/S, Q) that was collected 4 days later. The L-selectin/IgM chimera was purified and concentrated approximately 40-fold by affinity chromatography on goat anti-human IgM agarose.

Immunohistochemistry Procedures—Peripheral (axillary) and mesenteric lymph nodes and Peyer's patches were isolated from mice immediately after sacrifice. These lymphoid tissues were embedded in OCT medium (Tissue-Tek, MILES, Elkhart. IN), sectioned with a Leica 2800N cryostat, and collected on glass microscope slides.

Sections to be stained with anti-Fuc-TVII were fixed in 2% paraformaldehyde in phosphate-buffered saline for 20 min on ice. The sections were rinsed with phosphate-buffered saline at room temperature, were quenched with 50 mM NH_4Cl in phosphate-buffered saline at room temperature, and then were rinsed briefly with water. The tissues were then permeabilized with 100% methanol for 20 min on ice, rehydrated in phosphate-buffered saline, and then incubated for 30 min at room temperature with blocking solution A (phosphate-buffered saline containing 2% goat serum, 0.05% Triton X-100, 0.05% Tween 20). The blocking solution was aspirated, and the sections were incubated overnight at 7 °C with antigen affinity purified anti-Fuc-TVII used at a final concentration of 5 μg/ml in blocking solution A. After the overnight incubation, the anti-Fuc-TVII/blocking solution was removed, and the slides were washed with phosphate-buffered saline and were incubated for 1 h at room temperature with a FITC-conjugated goat anti-rabbit IgG reagent (Sigma) diluted 1:200 in blocking solution A. The slides were then washed at room temperature in phosphate-buffered saline, mounted with citifluor (Citifluor Products, Chemical Laboratory, The University, Canterbury, Kent, UK), and examined by immunofluorescence microscopy (Leitz DM RB microscope).

Sections to be stained with the monoclonal antibody MECA-79 (48) were fixed on ice for 20 min in 2% paraformaldehyde in phosphate-buffered saline, washed at room temperature with phosphate-buffered saline, and quenched for 20 min at room temperature with 50 mM NH_4Cl in phosphate-buffered saline. The slides were then rinsed briefly in water, permeabilized with 100% methanol for 20 min on ice, rehy-

drated in phosphate-buffered saline, and then incubated overnight at room temperature with blocking solution A (phosphate-buffered saline containing 2% goat serum, 0.05% Triton X-100, 0.05% Tween 20). The blocking solution was then aspirated, and the sections were incubated for 1 h at 7 °C with MECA-79 at a concentration of 5 μg/ml in blocking solution A. Sections were then washed extensively with phosphate-buffered saline at room temperature. The washed sections were then incubated for 1 h at room temperature with a tetramethyl rhodamine isothiocyanate-conjugated goat anti-rat IgM reagent (Jackson ImmunoResearch) used at a dilution 1:200 in blocking solution A. The slides were then washed three times at room temperature with phosphate-buffered saline, mounted with citifluor, and examined.

Sections to be stained with the L-selectin/IgM chimera were fixed in 1% paraformaldehyde and 0.1 M cacodylate, pH 7.1, for 20 min on ice, were then washed with Tris-buffered saline, pH 7.4. The L-selectin/IgM chimera was applied to the sections at a concentration 60 μg/ml in blocking solution B (Tris-buffered saline, pH 7.4, containing 2% goat serum) supplemented with either 3 mM $CaCl_2$ or with 5 mM EDTA, and were allowed to incubate overnight at 7 °C. Sections were then washed extensively with ice-cold Tris-buffered saline supplemented with 3 mM $CaCl_2$. Sections were then incubated for 1 h at 7 °C with a biotinylated goat anti-human IgM reagent (Sigma), diluted 1:200 in blocking solution B, and supplemented either with 3 mM $CaCl_2$ or with 5 mM EDTA. The sections were then washed with ice-cold Tris-buffered saline supplemented with 3 mM $CaCl_2$ and were incubated for 1 h at 7 °C with a FITC-conjugated streptavidin reagent (Vector Labs, Burlingame, CA) diluted 1:200 in blocking solution B supplemented with 3 mM $CaCl_2$. The slides were washed with ice-cold Tris-buffered saline supplemented with 3 mM $CaCl_2$, mounted with citifluor, and examined by immunofluorescence microscopy (Leitz DM RB microscope).

In Situ Hybridization Procedures—*In situ* hybridization procedures were completed using a modification of published procedures (59). Fresh murine axillary lymph nodes, mesenteric lymph nodes, and Peyer's patches were embedded in OCT medium (Tissue-Tek, MILES, Elkhart, IN) and quick-frozen in isopentane on liquid nitrogen. Cryostat sections (10 μM) were collected on Superfrost/Plus microscope slides (Fisher) fixed in freshly prepared 4% paraformaldehyde in PBS for 30 min on ice, washed twice in PBS, and digested for 5 min at room temperature with 1 μg/ml proteinase K in 50 mM Tris-HCl, pH 7.5, and 5 mM EDTA. The slides were then washed in PBS, fixed again in 4% paraformaldehyde, rinsed in water, and treated with 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0, for 10 min at room temperature. Acetylation was followed by room temperature washes in PBS and then in 0.85% NaCl. The slides were then dehydrated in a graded series of solutions of ethanol in water (30, 50, 80, 95, and 100% ethanol). Air-dried sections were overlaid with a hybridization solution containing ^{35}S-labeled RNA in sense or antisense orientation. RNA probes were derived by *in vitro* transcription procedures, using recombinant T7 or Sp6 RNA polymerases, initiating on the T7 or Sp6 promoter sequences flanking a DNA segment derived from the coding region of the mouse Fuc-TVII gene (base pairs 2197–2494; see Fig. 2) as subcloned into the vector pCDNAI (Invitrogen). The hybridization solution contained 50% deionized formamide, 0.3 M NaCl, 20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 10 mM phosphate buffer, pH 8.0, 10% dextran sulfate, 1 × Denhardt's solution (49), 0.5 mg/ml yeast tRNA, 10 mM dithiothreitol, and 10^7cpm/ml of radiolabeled probe. The hybridization solution was sealed over the sections with a coverslip and DPX mounting media (BDH Lab Supplies, Poole, UK). Hybridization was carried out for 16 h at 55 °C in a sealed container humidified with 5 × SSC. After hybridization, the DPX mounting media seal was removed, and slides were washed at 55 °C for 30 min in 5 × SSC and 10 mM dithiothreitol and then at 65 °C for 30 min in formamide wash buffer (50% formamide, 2 × SSC, 20 mM dithiothreitol). The slides were then washed 4 times at 37 °C in 0.5 M NaCl, 10 mM Tris-HCl, pH 7.5, 5 mM EDTA. Slides were then digested with RNaseA (1 μg/ml) for 30 min at 37 °C and were washed in formamide buffer, then in 2 × SSC, and then in 0.1 × SSC. Slides were dehydrated in a graded series of solutions of ethanol in 0.3 M ammonium acetate (30, 50, 80, 95, and 100% ethanol), air-dried, and coated with NTB2 liquid emulsion (Kodak). Following a 2–3-week exposure time, the emulsion was developed using procedures suggested by the manufacturer. Sections were then stained with hematoxylin and eosin and examined and photographed with bright field and dark field modalities, using a Leitz DM RB microscope.

RESULTS

A Hybridization Screen Identifies a Novel Murine α(1,3)Fucosyltransferase Locus—In an effort to isolate novel murine α(1,3)fucosyltransferase genes, a murine genomic DNA phage library was screened with a probe corresponding to the catalytic domain of the human Lewis α(1,3/1, 4)fucosyltransferase (Fuc-TIII) (21) using low stringency hybridization conditions (see "Experimental Procedures"). One phage was isolated that contained an insert with a translational reading frame sharing approximately 40% amino acid sequence similarity with the amino acid sequences encoded by four previously cloned members of the human α(1,3)fucosyltransferase family (Fuc-TIII-VI) (21–26).

To identify transcripts corresponding to this genomic sequence, a segment of the phage insert representative of the open reading frame was used to probe Northern blots prepared from mouse cell lines and tissues. Transcripts corresponding to this probe were identified in the murine cytotoxic T-cell line 14-7fd (42, 43). A cDNA library constructed from this cell line (32) was screened by hybridization with a segment of the phage insert, yielding 16 hybridization positive colonies. The sequences of all 16 cDNA clones were determined, as was the sequence of the corresponding genomic DNA (Fig. 1A). Analysis of this sequence data indicates that this locus yields multiple structurally distinct transcripts derived from alternative splicing events and possibly also from alternative transcription initiation events. Five classes of cDNAs were identified (Fig. 1). Analysis of these cDNA sequences identifies three methionine codons that may function to initiate translation of an open reading frame with amino acid sequence similarity to human Fuc-TIII, Fuc-TIV, Fuc-TV, and Fuc-TVI (Fig. 2). The positions of these methionine codons predicts the synthesis of α(1,3)fucosyltransferases with different cytosolic domains (encoded by exons 2 and/or 3) but with identical Golgi-localized catalytic domains (encoded by exon 4). One relatively abundant class of cDNAs (represented by cDNA 14) maintains an open reading frame initiating at the methionine codon at nucleotide 1947. This reading frame predicts a 342-residue, 39,424-Da type II transmembrane protein with a hydrophobic transmembrane segment derived from amino acids 9–31 (Fig. 2). An in-frame methionine codon at nucleotide 2126 predicts a 318-residue, 36,836-Da polypeptide that initiates within the hydrophobic transmembrane segment of the polypeptide predicted by the longer reading frame initiated at nucleotide 1947. A similar structural arrangement is found in two other cDNA classes, represented by cDNAs 6 and 10. However, these two cDNAs differ from cDNA 14 in that they contain an additional upstream exon with a methionine codon corresponding to nucleotide 996. The translational reading frame initiated by this methionine codon is truncated by a termination codon in exon 2 at a position proximal to the methionine codon at nucleotide 1947 and thus cannot generate a polypeptide that shares similarity to the human α(1,3)fucosyltransferases. However, in cDNA 5, the absence of exon 2 allows the translational reading frame generated by the methionine codon at nucleotide 996 to continue in frame with sequence in exon 4. This arrangement predicts the synthesis of a 389-residue, 44,492-Da type II transmembrane protein with the same putative transmembrane segment defined for the protein predicted by cDNA 14 (Fig. 2). Finally, cDNA 3 is representative of a relatively abundant class of cDNAs that each initiate between the splice acceptor site of exon 4 and the methionine codon at nucleotide 2126. This class of cDNAs predicts a 318-residue, 36,836-Da polypeptide that initiates within the transmembrane segment predicted for the proteins corresponding to the other cDNA classes.

Because the polypeptides predicted by these murine cDNAs share primary sequence similarity to the four human α(1,3)fucosyltransferases known at the time (Fuc-TIII, IV, V, and VI), we anticipated that one or more of them would function as an

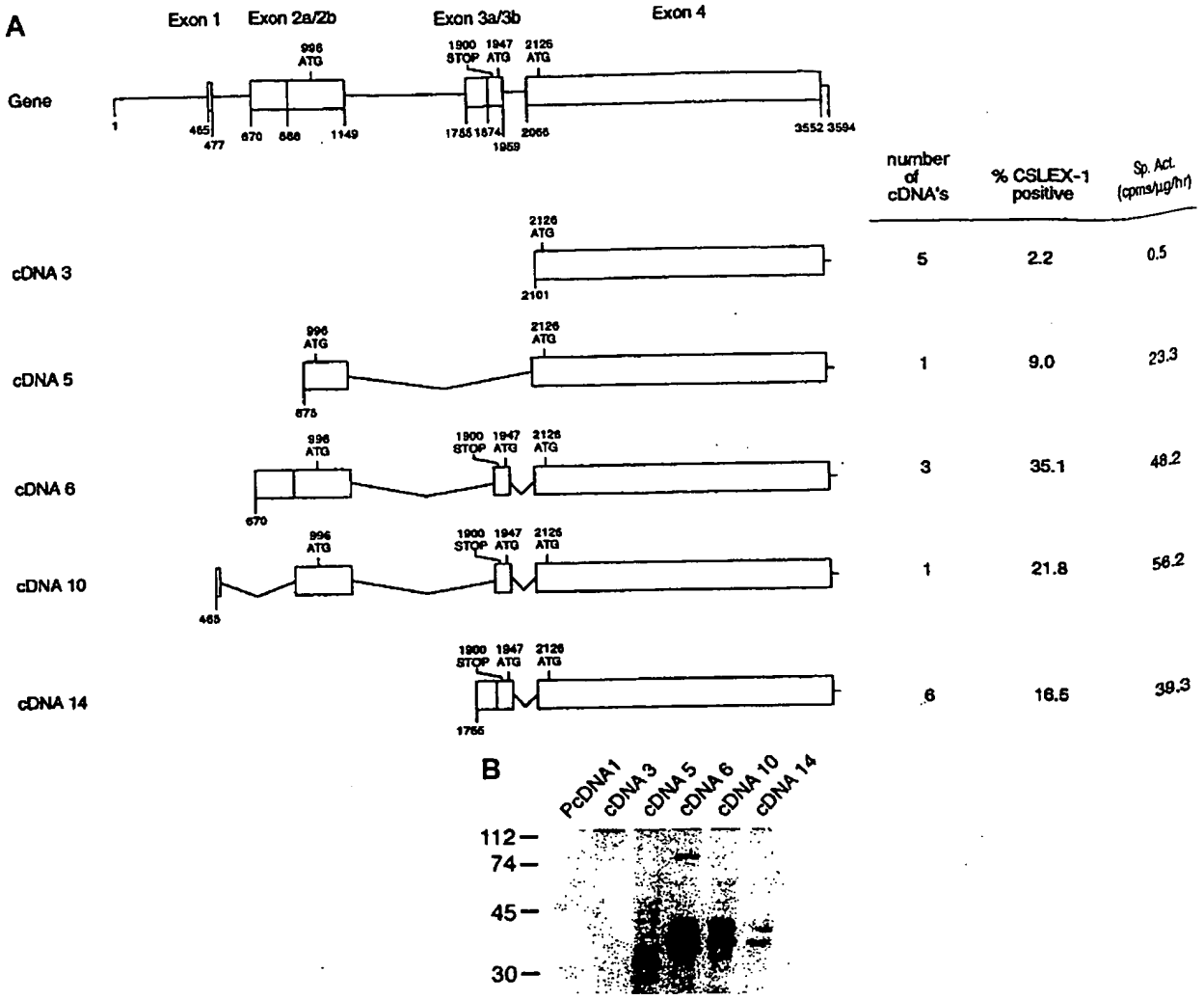

FIG. 1. **Structure and function of the murine Fuc-TVII gene and cDNAs.** *A*, structures and functional activities of the murine Fuc-TVII gene and cDNAs. The multi-exon structure of the murine Fuc-TVII gene is shown at the *top*. Numbering *below* the schematic corresponds to the nucleotide positions of intron-exon boundaries, and the first (1) and last (3594) nucleotides of the known sequence of the locus. Intron-exon boundaries are defined by comparison of the cDNA sequences to the corresponding genomic DNA sequence (see Fig. 2). The numbering *above* the *schematic*, immediately *above* the *ATGs*, corresponds to the nucleotide position of the first nucleotide in each of the three potential initiation codons as discussed in detail in the text. The numbering *above* the *schematic*, immediately *above* the *STOPs*, corresponds to the nucleotide position of the translational termination codon (TGA; base pairs 1900–1902) localized to exon 3b that truncates the potential open reading frame initiated by the start codon at nucleotide 996–998 in cDNA classes represented by cDNAs 6 and 10 (see text for details). Representative members of the five structurally different classes of Fuc-TVII cDNAs isolated from the murine cytotoxic T-lymphocyte cell line 14-7fd are schematically represented *below* the gene structure *schematic*. The cDNAs shown are the representative member of each class with the longest 5′ extension. The number of cDNAs isolated in each class is indicated in the column labeled *number of cDNA's*. Each cDNA was transiently expressed in COS-7 cells (see "Experimental Procedures"). The transfected COS-7 cells were then subjected to flow cytometry analysis to characterize the cell surface glycosylation phenotype determined by each cDNA. The fraction of sialyl Lewis x-positive cells in the transfected population (positive staining with the monoclonal antibody CSLEX-1, normalized to transfection efficiency as determined by chloramphenicol acetyltransferase activity encoded by a co-transfected plasmid vector encoding this enzyme ("Experimental Procedures") is indicated in the column labeled *% CSLEX-1 positive*. These results represent the fraction of antigen-positive cells observed above a background of 2% staining obtained with the negative control vector pCDM8. Extracts were also prepared from the transfected cells and were subjected to *in vitro* α(1,3)fucosyltransferase assays using 5 mM sialyl *N*-acetyllactosamine as an acceptor (see "Experimental Procedures"). The specific activity of the α(1,3)fucosyltransferase activity encoded by each cDNA (normalized for transfection efficiency) is indicated in the column labeled *Sp. Act. (cpms/μg/hr)*. *B*, Western blot analysis of the polypeptides expressed in COS-7 cells by cDNAs 3, 5, 6, 10, and 14. The extracts used in the α(1,3)fucosyltransferase assays discussed in *A* were also subjected to Western blot analysis using an antigen affinity purified anti-FucTVII antibody. The amounts of protein analyzed from each type of transfected cell extract were varied to achieve normalization to the transfection efficiencies, exactly as indicated in *A* for the flow cytometry and α(1,3)fucosyltransferase activity analyses. Cell extracts were fractionated by SDS-polyacrylamide gel electrophoresis and electroblotted to a polyvinylidene difluoride membrane, and the Fuc-TVII expression vector-encoded polypeptides were identified by probing with an antigen affinity purified rabbit anti-Fuc-TVII antibody, goat anti-rabbit IgG-peroxidase conjugate, and a commercially available enhanced chemiluminescence reagent (ECL, Amersham Corp.) as described under "Experimental Procedures."

α(1,3)fucosyltransferase. However, because the murine peptide sequence shares approximately equivalent sequence similarity to each of these human enzymes, we expected that it did not represent the murine homologue of any of them and consequently named it Fuc-TVII. This appellation has been justified by subsequent work in which this murine gene has been used to isolate cDNAs encoding the human Fuc-TVII (27).

None of the three putative initiation codons are embedded in

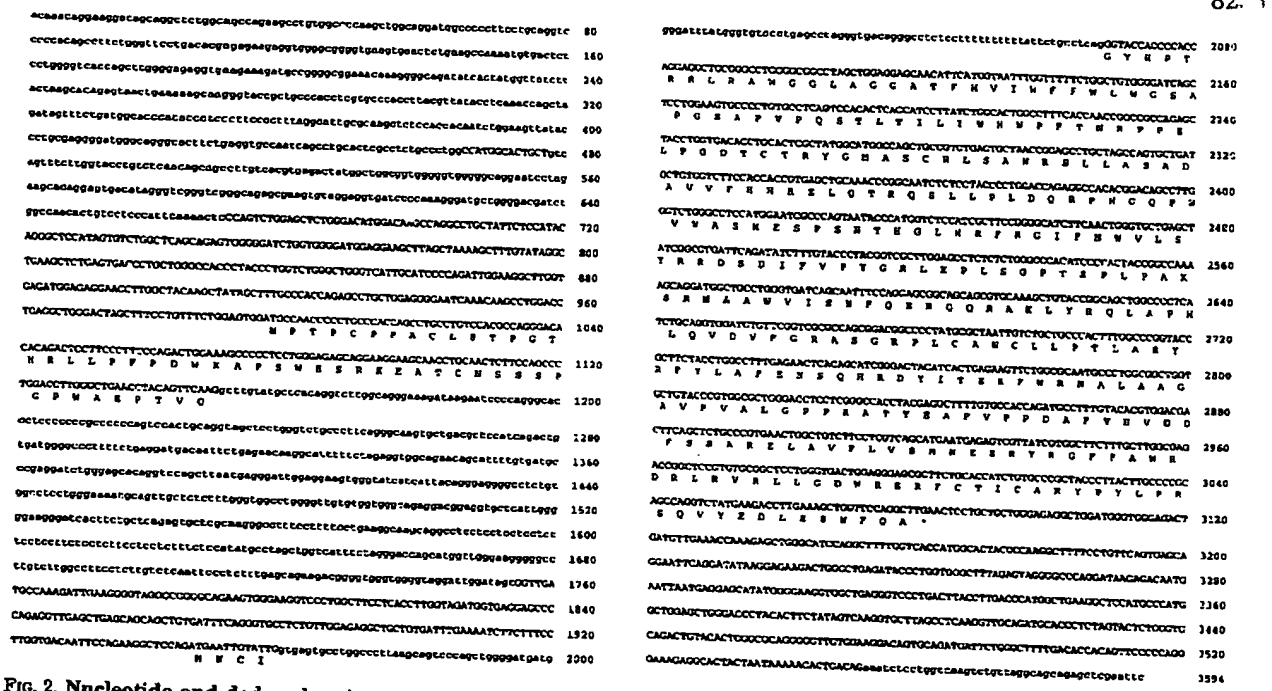

FIG. 2. **Nucleotide and deduced amino acid sequence of the isolated mouse Fuc-TVII gene.** The DNA sequence was derived from a phage containing the murine Fuc-TVII locus. The DNA sequence present in cDNAs (Fig. 1) is shown in *uppercase letters*, whereas the DNA sequence corresponding to intronic positions is displayed in *lowercase letters*. Amino acid sequences predicted by the cDNA sequences are shown in single-letter code. As discussed in detail in the text, alternative splicing events yield different cDNAs that may in turn encode three different polypeptides. One protein is predicted to initiate at the methionine codon localized to nucleotide positions 996–998 (389 residues, 44,492 Da; cDNA 5; Fig. 1). A second protein is predicted to initiate at the methionine codon localized to nucleotide positions 1947–1949 (342 residues, 39,424 Da; cDNAs 6, 10, and 14; Fig. 1). The third protein is predicted to initiate at the methionine codon localized to nucleotide positions 2126–2128 (318 residues, 36,836 Da; cDNA 3, as well as all other cDNAs; Fig. 1).

a sequence context consistent with Kozak's rules for translation initiation (Fig. 2) (60). To determine which, if any, of these initiation codons and cognate cDNAs function to encode the predicted polypeptide(s) and to confirm that this locus encodes an α(1,3)fucosyltransferase, COS-7 cells were transfected with a cDNA representative of each class, and the transfectants were subjected to assays to (i) identify cDNA-determined cell surface-localized fucosylated oligosaccharide antigens, (ii) identify and quantitate the polypeptides encoded by cDNAs, and (iii) identify and partially characterize cDNA-determined α(1,3)fucosyltransferase activity in transfectant cell extracts using *in vitro* α(1,3)fucosyltransferase activity assays.

cDNAs representative of three of the five classes (cDNAs 6, 10, and 14) (Fig. 1) each determine relatively high levels of cell surface-localized sLe^x expression (35.1, 21.8, and 16.5%, respectively, above a 2% background) when introduced into COS-7 cells by transfection. cDNA 5 also directs cell surface sLe^x expression in COS-7 cells but at a level (9% positive cells) that is lower than the sLe^x expression levels determined by cDNAs 6, 10, and 14. By contrast, none of these four cDNAs directs expression of Lewis x, Lewis a, or sialyl Lewis a determinants (data not shown). These results indicate that one or both of the two potential methionine initiator codons in each cDNA can efficiently direct translation to yield α(1,3)fucosyltransferase activity. These observations further indicate that this α(1,3)fucosyltransferase activity can utilize α(2,3)sialylated lactosamine-based glycan structures to form sLe^x determinants but indicate that the activity does not efficiently utilize neutral type II oligosaccharide Lewis x precursors nor neutral or α(2, 3)sialylated type I precursors to the Lewis a isomers. Because all four of these cDNAs direct qualitatively identical cell surface antigen profiles in COS-7 cells, it seems likely that individually or together, each directs the expression of polypeptides that individually or together maintain essentially identical acceptor substrate specificities (at least for the four antigens examined).

In contrast to the results obtained with cDNAs 5, 6, 10, and 14, cDNA 3 does not direct detectable sLe^x expression. This result suggests that the methionine codon at nucleotide 2126 in this cDNA does not efficiently promote initiation of translation of the cognate mRNA and thus does not encode functionally significant levels of enzyme activity. Alternatively, this cDNA may encode a polypeptide without α(1,3)fucosyltransferase activity.

Qualitatively identical results were obtained when these five cDNAs were expressed in another cell line (CHO-Tag cells) (32) informative for expression of the Lewis x and sLe^x determinants (data not shown). Unlike COS-7 cells, this cell line is also capable of forming the internally fucosylated VIM-2 determinant (NeuAcα2,3Galβ1,4GlcNAcβ1,3Galβ1,4(Fucα1,3) GlcNAc-R) (22). We found that none of the cDNAs directs expression of the VIM-2 epitope when expressed in the CHO-Tag cells (data not shown). Considered together, these results indicate that some, though not all, of the cDNAs can encode an α(1,3)fucosyltransferase activity that can catalyze α(1,3)fucosylation of the *N*-acetylgalactosamine moiety on a terminal α(2,3)sialylated lactosamine unit but not to internal *N*-acetylgalactosamine moieties on α(2,3)sialylated polylactosamine precursors nor to neutral type II precursors.

To confirm that the sLe^x expression efficiency characteristic of each cDNA correlates with the level of expression of the corresponding protein, cell extracts of the transfected COS-7 cells were subjected to Western blot analysis using an affinity purified rabbit polyclonal antibody generated against a recombinant form of the predicted polypeptide (Fig. 1*B*). Cells transfected with cDNAs 6, 10, and 14 express two major forms of the

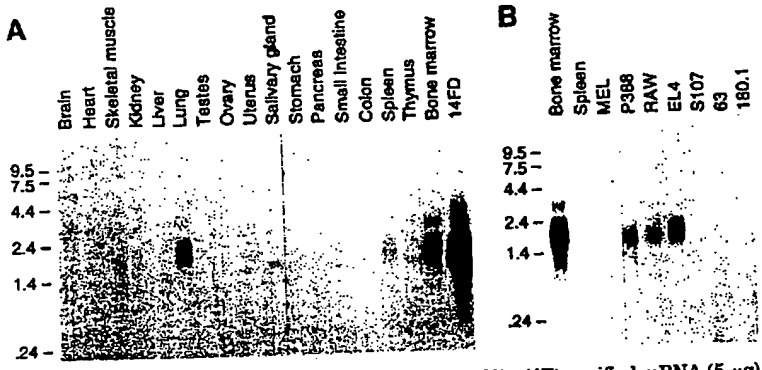

FIG. 3. **Tissue-specific expression patterns of the murine Fuc-TVII gene.** Oligo(dT) purified mRNA (5 μg) purified from murine tissues and cell lines was fractionated by agarose gel electrophoresis, blotted to a nylon hybridization membrane, and probed with a 974-base pair DNA segment derived from the coding region of the mouse Fuc-TVII locus (nucleotides 2228–3207; see Fig. 2 and "Experimental Procedures"). RNA molecular size standards, in kilobase pairs, are indicated at the *left* in each panel. Each blot was subsequently stripped and reprobed with a radiolabeled chicken glyceraldehyde 3-phosphate dehydrogenase probe to confirm that RNA samples were intact and loaded in equivalent amounts (see "Experimental Procedures," data not shown). *A*, polyadenylated RNA isolated from mouse tissues and from the murine T-lymphocyte cell line 14-7fd (*14FD*). *B*, polyadenylated RNA isolated from mouse bone marrow and spleen and from cultured murine leukocyte cell lines. Cell lines represent the following lineages: *MEL*, murine erythroleukemia cell line; P388 and RAW (RAW 264.7), macrophage; *EL4*, T cell; S107, 63 (TH2.54.63) and 180.1, B cell lines (hybridomas).

protein, with molecular masses of 35 and 37 kDa. Smaller amounts of several other proteins are also evident in these cells. The amount of immunoreactive protein generated by these three cDNAs correlates with the level of sLe^x expression directed by each. This observation indicates that the relative sLe^x expression level directed by each is a function of the efficiency with which each corresponding mRNA is translated and thus the relative intracellular accumulation of the cognate polypeptide.

Cells transfected with cDNA 5 cells also contain multiple immunoreactive polypeptides (Fig. 1*B*). The most abundant pair of these proteins migrate more rapidly than do the proteins detected in cells transfected with cDNAs 6, 10, and 14 yet are approximately similar in quantity to the immunoreactive protein directed by cDNAs 6 and 10. Because cDNA 5 directs lower levels of cell surface sLe^x expression than these two cDNAs, it is therefore possible that the lower M_r immunoreactive polypeptides found in cDNA 5-transfected cells maintain substantially lower specific enzyme activity than do the proteins encoded by cDNAs 6, 10, and 14 or are otherwise less able to direct sLe^x expression in COS-7 cells. Finally, cells transfected with cDNA 3 do not contain any detectable immunoreactive proteins. This implies that the putative initiator codon at base pair 2126 in this cDNA does not initiate translation of an immunoreactive product and is consistent with the observation that this cDNA does not yield sLe^x expression following transfection into COS-7 cells.

Conclusions derived from the flow cytometry and Western blot analyses summarized above are supported by the results of *in vitro* α(1,3)fucosyltransferase assays completed on the same cell extracts. These assays demonstrate that cells transfected with cDNAs 5, 6, 10, and 14 contain enzyme activity that can transfer ^{14}C-labeled fucose from the nucleotide donor substrate GDP-fucose to the low molecular weight acceptor 3′-sialyl *N*-acetyllactosamine (NeuNAcα2, 3Galβ1, 4GlcNAc) (Fig. 1*A*). For each cDNA, the product of this reaction co-elutes with a radiolabeled sLe^x tetrasaccharide standard when fractionated by ion suppression amine adsorption high pressure liquid chromatography (data not shown). The α(1,3)fucosyltransferase activity directed by each of these four cDNAs does not utilize the neutral acceptor substrates *N*-acetyllactosamine or lacto-*N*-biose I. Extracts prepared from cells transfected with cDNA 3 do not contain detectable α(1,3)fucosyltransferase activity when tested with 3′-sialyl *N*-acetyllactosamine nor when tested with the neutral acceptor substrates *N*-acetyllactosamine or lacto-*N*-biose I. These results are entirely consistent with the flow cytometry data summarized above and indicate that this locus encodes an α(1,3)fucosyltransferase activity that apparently requires type II acceptor substrates that are terminally substituted with an α(2,3)-linked sialic acid residue. Considered together, these results suggest that differential splicing and/or transcriptional initiation events can control the level of α(1,3)fucosyltransferase activity and thus cell surface sLe^x expression level through mechanisms that depend on the efficiency with which each transcript is translated.

Transcription of the Mouse Fuc-TVII Locus Is Restricted to Cells Found in the Bone Marrow and the Lung—Northern blot analysis indicates that transcripts corresponding to the Fuc-TVII locus accumulate to detectable levels in only a few tissues in the adult mouse (Fig. 3). Abundant transcript accumulation is only observed in the lung and in the bone marrow, with very small amounts evident in the spleen, salivary gland, and skeletal muscle. Northern blot analysis of cultured murine blood cell-type cell lines indicates that the Fuc-TVII transcript is relatively abundant in the mouse cytotoxic T line 14-fd (used to clone the Fuc-TVII cDNAs) and in the mouse T cell line EL4 (34). Less abundant transcript accumulation is evident in the murine macrophage-derived lines RAW (39, 40) and P388 (41). Fuc-TVII transcripts are not evident in the murine erythroleukemia cell line MEL (37, 38) nor in three murine B-lymphocyte lineage cell lines (S107 (33); TH2.54.63 (35); 180.1 (36)).

Both the marrow and lung maintain several differently sized transcripts, including two abundant transcripts of approximately 1.6 and 2.2 kilobase pairs in size and a fainter transcript at approximately 3.0 kilobase pairs. These three transcripts are similar in size to the three most abundant transcripts observed in the murine 14-7fd cytotoxic T cell line. These observations suggest that cells in the bone marrow and lung yield alternatively spliced transcripts similar in structure to those characterized by cDNA cloning studies in the 14-7fd cells. These data also suggest that in the marrow, the Fuc-TVII locus is transcribed in cells assigned to the myeloid and T-lymphoid lineages but not in B-lymphoid lineage cell types and suggest that expression of this fucosyltransferase correlates with selectin ligand expression on myeloid and T-lymphocyte lineage cell types.

Fuc-TVII Is Expressed in Endothelial Cells Lining the High Endothelial Venules in Peripheral Lymph Nodes, Mesenteric